Fabrication and Characterization of Electrodes Functionalized with Enzymes and Mediators

氏名	いすらむ　もはめど　かむるる ISLAM MD KAMURUL
授与学位	博士（工学）
学位授与年月日	平成17年9月14日
学位授与の根拠法規	学位規則第4条第1項
研究科，専攻の名称	東北大学大学院工学研究科（博士課程）生物工学専攻
学位論文題目	Fabrication and Characterization of Electrodes Functionalized with Enzymes and Mediators
指導教員	東北大学教授　末永　智一
論文審査委員	主査　東北大学教授　末永　智一　　東北大学教授　熊谷　泉 東北大学教授　西澤　松彦　　教授

論文内容要旨

Diabetes mellitus has emerged as one of the life threatening diseases to human in the current era. Up until now, the disease is thought to be incurable and, hence, proper maintenance is the only way to cope with the diabetic hazards. Information about the exact blood glucose level is a prerequisite for the proper maintenance of diabetes. Real time information has been proven advantageous over the discrete blood sampling because discrete information often fails to deliver complete idea about blood glucose level; i.e., it is likely to miss hypo or hyperglycemic episodes.

Electrochemical biosensors have been considered to be one of the most suitable devices due to their selectivity, fast response, miniature size, reliability and reproducibility in the determination of various blood analytes. In this connection, the fabrication of glucose sensor is an important area of research in the diagnosis and management of diabetes. This sensor is very rapid, convenient and precise in the determination of blood and urine glucose level. In the recent years, a number of strategies have been undertaken to construct glucose sensor. To date, the most commonly used amperometric glucose sensor uses glucose oxidase (GOD) enzyme. At present, one of the commercially available glucose sensors is based on this enzyme. The sensor detects blood glucose indirectly by electrochemically determining the amount of hydrogen peroxide produced during its operation, which has made the device oxygen-dependent. The variation in the partial pressure of oxygen in blood often gives false readings. Also the redox potential of hydrogen peroxide is much positive (0.6V~0.7V vs. Ag/AgCl). Therefore, the sensor has a greater chance to be affected by the interfering agents present in the blood like acetaminophen, ascorbic acid etc. Steps have been taken to overcome this problem. These include use of osmium-based mediator for the relay of electrons directly from enzyme active site to the electrode. The drawback of this system is the toxic effect of osmium to human health.

Considering these facts, a different strategy of glucose sensor fabrication was undertaken. In this strategy, some points were strictly considered. First, reduction in the overpotential to avoid the hazards of interferents; second, use of glucose dehydrogeanse (GDH) enzyme which is oxygen insensitive; and third,

use of hazardless mediator. It is known that glucose dehydrogenase (GDH) is one of the NADH-dependent dehydrogenases. Therefore, the system is based on use of NADH. To oxidize NADH, diaphorase (Dp) is needed but this enzyme, like most redox enzymes, transfer electrons towards electrode surface slowly. This constraint is alleviated by the use of mediators which shuttles electrons from redox site to electrode with a relatively higher rate. The direct oxidation of NADH requires high overpotential which is compensated by the application of appropriate mediator. The reaction principle used here was also applied in biofuel cell anode because NAD^+/NADH couple is a cofactor for several hundred dehydrogenases and thereby the system could use varieties of fuel substrates present in body fluids for the purpose of power generation.

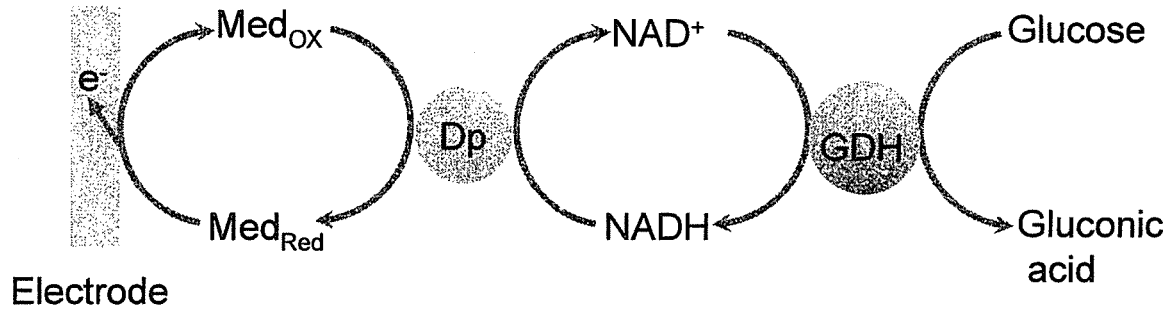

Fig. Reaction principle of the proposed glucose sensor

By far many of the mediators have been studied. Quinones are one of the most studied functional groups and play a vital role in electron transport chains of living organisms. In this work, the reaction kinetics between diaphorase and several mediators was studied and it was found that the rate constant of 2-methyl-1,4-naphthoquinone (VK3) was high. The redox potential of VK3 is also negative (-0.25V) which is one of the important characteristics of a good mediator. Since VK3 is not easy to be immobilized on electrode surface, its derivatives, 2-methyl-3-3[3-(2-aminoethyl-aminocarbonyl) propyl]-1,4-naphthoquinone (AEACPVK3), 5-hydroxy-1,4-napthoquinone (HNQ), and 5,8-dihydroxy-1,4-naphthoquinone (DHNQ) were used as mediator. AEACPVK3 possesses amino group which helps in its easy immobilization. HNQ and DHNQ were immobilized through their electro-polymerization. The AEACPVK3-bienzyme immobilized glassy carbon electrode showed good response to glucose oxidation. Both HNQ and DHNQ were immobilized with diaphorase and glucose dehgydrogease onto gold electrode. In this case, electrochemical immobilization of quinone was performed over bienzyme immobilized electrode by using organic solvent (acetonitrile). DHNQ-bienzyme was put into amperometric glucose sensing. The system was saturated at 4 mM glucose but lost its response in short time. This might be due to deactivation of enzyme by organic solvent during quinone immobilization.

The work was further extended to the application of thionine as mediator because thionine can directly oxidize NADH in absence of diaphorase. Therefore, the system could be simpler than the quinone based

sensor. Thionine was immobilized on a gold electrode via self assembled monolayer of 3,3'-dithiobis (succinimidyl propionate). The immobilized thionine produced two pairs of redox peaks at negative (-0.05V) and positive (+0.21V) potential. The redox waves at negative potential are thought to be of monomeric thionine; whereas, the redox waves at positive potential is due to dimeric thionine. The dissolved thionine which showed only one pair of redox waves at -0.14V was capable of oxidizing NADH. However, when it was immobilized, only the dimeric form showed NADH oxidizing capability. Glucose oxidation was investigated at the thionine immobilized electrode. The electrode was not able to oxidize glucose in presence of NADH and GDH.

The influence of gold nanoparticles was studied in this experiment. Thionine was immobilized on a nanoparticle supported gold electrode. This electrode showed a larger peak height at diemric redox potential site than in the case of thionine immobilized electrode. Again, the current for NADH oxidation at this electrode was 1.7 times greater compared to thionine immobilized electrode, which might be due to excess dimeric thionine deposition with the aid of nanoparticles. Interestingly, the nanoparticle supported thionine immobilized electrode showed response to glucose oxidation and hence glucose dehydrogenase was further immobilized onto this electrode. Amperometric glucose sensing was performed in a physiological solution containing 2 mM NAD^+ with the successive addition of glucose. The electrode showed increase in current up to addition of 2.5 mM glucose. The plot for oxidation current vs. glucose concentration corresponded to a Michaelis-Menten curve.

As a final approach, the NADH-dependent dehydrogenase system was applied to the anode of a biofuel cell where glucose was used as fuel substrate. The cathode reaction was simply the oxygen reduction. Three different mediators, AEACPVK3, anthraquinone-sulfonate (AQS) and polyallylamine-VK3 (PAA-VK3) were immobilized at an anode electrode and their performance was investigated in the output of cell power.

AEACPVK3 with diaphorase and glucose dehydrogenase was immobilized on a glassy carbon electrode. Before applying this electrode in a biofuel cell, potentiometric measurement was performed using this electrode in a three electrode system where the background solution was PBS (pH 7.0) containing 1 mM NADH and 5 mM glucose. Potential was measured between the quinone–bienzyme immobilized electrode (working electrode) and an Ag/AgCl reference electrode. The counter electrode was a coiled platinum wire. Upon application of 50 nA /cm^2 current, working electrode was able to keep the potential at 0.3 V which almost resembles the redox potential of AEACPVK3. But, when the value of applied current was 5 μA /cm^2, the potential shifted and was 0.2 V. With the further increase in current amplitude of 50 μA /cm^2, the potential at once shifted and the system was no longer be able to keep the working electrode potential. The ground behind this result could be explained as (a) inefficiency of the mediator molecules to direct electrons toward electrode because of their faulty orientation inside hydrogel and (b) failure in the expected diffusion of charge from substrate to inner biomolecules in the hydrogel, which might be a consequence of impermeable film.

A biofuel cell was constructed by using salt bridge between anode and cathode chamber. The anode chamber was made of PBS containing 0.2 mM NADH and 5 mM glucose and the cathode chamber was made only of PBS. The anode was AEACPVK3-bienzyme immobilized glassy carbon electrode and the

cathode was a platinum wire. The maximum power output obtained in this cell was 1.35 $\mu W/cm^2$ at 0.22V corresponding to 6.13 $\mu A/cm^2$.

Anthraquinone–sulfonate (AQS) was used in biofuel cell because it shows much negative potential which is very important characteristic of a mediator used in biofuel cell. The main hindrance in using this mediator is the difficulty in its immobilization onto electrode surface. Therefore, the performance of this mediator in biofuel cell was investigated by its application in dissolved state. Potentiometric measurement was carried out using a gold electrode immobilized by diaphorase and glucose dehydydrogeanse. The background solution was PBS containing 0.1 mM AQS, 0.2 mM NADH and 5 mM glucose. During 250 nA/cm^2 and 2.5 $\mu A/cm^2$ current applications, the working electrode was able to keep the potential at 0.42 V which almost resembles the redox potential of AQS. But in case of 25 $\mu A/cm^2$ current, the potential at once shifted and the system was no longer be able to keep the working electrode potential.

With an aim to increase in the charge transfer to the electrode, one more strategy was undertaken. This was based on a diaphorase/glucose dehydrogenase double layer-coated glassy carbon anode and a polydimethylsiloxane (PDMS)-coated Pt cathode. The inner diaphorase layer was prepared by co-immobilization with the newly synthesized PAA-VK3. A conductive support, Ketjenblack (KB) was applied in electrode modification. At the final step in immobilization process, glucose dehydrogenase was applied on the immobilized VK3-diaphorase membrane. A membraneless biofuel cell was constructed using functionalized anode and PDMS coated cathode in PBS containing 0.5 mM NADH and 10 mM glucose. The maximum power density was 14.5 $\mu W/cm^2$ at 0.36 V corresponding to 40.3 $\mu A/cm^2$. From this observation, it could be assumed that use of conductive support helps increase the power output of the cell.

The proposed work is posed with an aim to avoid some practical hazards being experienced during the use of currently available GOD–based in vivo glucose sensor. The use of NADH is a drawback of this proposed system because immobilization of NADH on electrode is another factor in the construction of the device. In fact this is merely a preliminary study of the targeted device. Undoubtedly it could be said that the principle of this system is very much promising from practical viewpoint. To reach the goal, it is required to proceed step by step and hence it demands repeated, thorough investigations.

論文審査結果の要旨

本学位論文は，酵素およびメディエーターを同時に固定化した電極を作製，評価し，バイオセンサおよび生物燃料電池へ適用した研究についてまとめたものである．酵素としてデヒドロゲナーゼを利用しているため，センサにおける測定対象物質および生物燃料電池における燃料の対象を拡充できるシステムである．メディエーターとしてナフトキノン類およびフェノチアジン類を選択しており，夾雑物質の影響を排除できる電位での計測に成功している．また，酵素およびメディエーターを固定化した電極は，測定対象溶液（血液，体液および尿など）に添加物を加えることなく計測が可能である．本論文は全6章から構成されており，下記に章ごとに概説した．

第1章は序論であり，本研究の背景および目的について述べている．

第2章では，本研究で用いた電気化学計測法および電極の作製法等について述べている．

第3章では，固定化担体であるポリビニルイミダゾール（PVI）を，架橋剤を介して酵素およびメディエーターで修飾し，電極表面上への一括固定を達成している．酵素には，NADH酸化酵素であるジアフォラーゼおよびグルコースデヒドロゲナーゼ（GDH）を用いている．NADHおよびグルコースの添加に伴うナフトキノン誘導体の触媒酸化電流の増加が観測された．グルコース濃度に依存した電流応答を調査し，センサとして機能することを確認した．また，架橋剤の種類および濃度依存性を検討し，センサ機能の向上を目指して最適化した．

第4章では，メディエーターとしてフェノチアジン類であるチオニンを適用した．チオニンを固定化した金ナノ微粒子とGDHを電極表面上に固定化し，グルコース濃度に依存した触媒酸化電流応答を得た．金ナノ微粒子固定化電極を利用することにより，反応電極表面の増加および触媒活性を有するフェノチアジン二量体濃度の増加が認められ，効率的にグルコースが酸化できることを示した．この電極を用いることにより，高感度で再現性に優れたグルコースセンサを作製した．

第5章では，第3章で作製した酵素およびメディエーター固定化電極をアノードとした生物燃料電池を開発している．白金カソードと組み合わせ，グルコースを燃料とした生物燃料電池を作製し，その特性を明らかにした．アノードにおける酸素除去にグルコースオキシダーゼ膜を用い，カソードに酸素選択性膜を適用することによりセパレータフリーの生物燃料電池を構築した．

第6章は以上を総括している．

以上，本論文は，酵素およびメディエーターを同時固定した電極を開発するとともに，これを利用したバイオセンサおよび生物燃料電池に関してまとめたものであり，生物工学の発展に寄与することが少なくない．

よって，本論文は博士（工学）の学位論文として合格と認める．